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A cone-dominant retina system is being developed for examining the cellular basis of ischemic damage. Normal cGMP levels in the chick retina decreases in the light. cAMP changes little. The sightless mutant rd has low levels cGMP and high levels of cAMP. Ischemic injury in both normal and rd chicks causes increased levels of both cyclic nucleotides and loss of light sensitivity. Localization of associated enzymes was also done.

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Introduction

It is well known that the central nervous system is very sensitive to trauma induced hypoxia and ischemia. The photoreceptor cells of the retina are among the more susceptible to ischemic injury and, in humans, among the earliest discernible signs of such injury.

In the vertebrate retina, photoreceptor cell function, cyclic nucleotide metabolism and metabolic activity are tightly coupled and are jointly regulated by light. Within the photoreceptor cell, the conversion of light into a neural signal (phototransduction) is mediated by light-induced decreases in the outer segment concentration of cGMP (Stryer, 1986). cAMP acts as a second messenger in synaptic transmission between neurons in the inner retina. The retina has the highest metabolic rate of any tissue measured (Warburg, 1927). The primary consumers of oxygen and glucose in the retina are the photoreceptor cells (Zuckerman and Weiter, 1980, Cohen, 1965), which require large amount of energy to support phototransduction.

This large energy demand of the photoreceptors contributes to their susceptibility to ischemic insult. Ischemia rapidly disrupts the function and structure of photoreceptor cells, affecting vision mediated by both rod and cone photoreceptor cell types (Ames and Gurian, 1963, Webster and Ames, 1965). Little is known, however, about the cellular changes in cone cells during and after ischemia. Cone photoreceptors subserve vision high in spatial and temporal resolution, allow color perception and are the only photoreceptor present in the fovea, the area of highest visual acuity in man. Determining the extent to which ischemia alters cyclic nucleotides and energy charge in cone cells and their neuronal networks may provide necessary information useful for establishing preventive measures in ischemic trauma, retinopathies and surgery.

Our laboratory is employing the chicken retina as a model system in which to study the cellular and subcellular effects of ischemia on cone photoreceptors. Approximately 90% of the photoreceptors in the central portion of the chicken retina are cones (Morris and Shorey, 1967). The chicken retina is well characterized in terms of development, morphology and electrophysiology and is fully differentiated and functional at the time of hatching. The orderly laminar arrangement of various cell types and cellular regions within the chicken retina make it very conducive to the microdissection of individual cell layers for cyclic nucleotide analysis. In addition, the rd (retinal degenerate) chick, which possesses a nontransducing yet morphologically normal retina at hatching, is being employed to compare the effects of ischemia on cyclic nucleotides in the presence and absence of function. The rd Rhode Island Red strain of chicken possesses an autosomal recessive mutation which results in blindness at hatch followed 7-10 days later by the onset of progressive rod-cone degeneration (Ulshafer and Allen, 1985). Chicks heterozygous for the mutation (carriers) retain normal vision and retinal morphology throughout their lives.

For a meaningful study of the effects of ischemia on in vivo levels of cyclic nucleotides, it is imperative that accurate baseline values of nonischemic cyclic nucleotide levels first be established.

This we found to be a fairly major undertaking since it is not obvious what would constitute a firm baseline. A great deal of effort was expended the first year to develop this baseline as unambiguously as possible. The technique chosen must reproducibly inactivate retinal metabolism as quickly as possible with minimal confounding factors. Anesthesia, stress and trauma are known to rapidly alter cyclic nucleotide levels in neuronal tissue (Nemoto, 1982). The inactivation method employed (i.e., freezing, microwave irradiation) should also be tested as a possible source of artifactual change in cyclic nucleotide levels.

Freezing, for example, has been reported (Farber, 1981) to selectively decrease cAMP but not cGMP levels in retinas of certain cone-dominant species. Therefore, retinal studies which employ freezing as a means of tissue inactivation must be tested for the possibility of freeze-induced changes in cyclic nucleotide levels.

Objectives

The overall objective of the present study was to begin to examine the effects of ischemia on cyclic nucleotide metabolism in the cone-dominant retina with respect to the light-adapted state and functional condition of the retina. Specifically, the following questions were addressed in the first year:

1. Can a viable model for trauma induced ischemic damage to a cone-dominant retina and its photoreceptors be developed?
2. What are the normal baseline or nonischemic concentrations of cGMP and cAMP in the cone-dominant chicken retina and how are they affected by light?
3. Does inactivation of retinal metabolism by freezing alter cyclic nucleotide levels in the chick retina?
4. How do ischemic levels of cyclic nucleotides compare with nonischemic levels in the sighted chick retina?
5. Where are cAMP and cGMP concentrated within the nonischemic chick retina?
6. Is the distribution, light modulation and ischemic response of cyclic nucleotide levels comparable in the sighted chick retina and the nonfunctioning but morphologically normal rd retina?

Methods

The ischemic state

The standard procedure for inducing ischemia was by decapitation, and

processing in cold acid or in liquid N_2. The standard nonischemic state developed is decapitation followed by immediate immersion in liquid N_2.

Light modulation of cGMP and cAMP levels in nonischemic and ischemic retinas of sighted and rd chicks

Sighted carrier chicks and blind rd chicks, 1-2 days posthatch, were either dark-adapted in total darkness or light adapted in laboratory illumination (approximately 150 ft. cd.) for 2-3 h. Under the same lighting conditions, chicks were sacrificed by decapitation. For the ischemic protocol, chicks were enucleated, the anterior segment of each eye removed with a razor blade and the vitreous extracted. The retina-pigment epithelium-choroid complex was then teased from the eyecup and either frozen in liquid nitrogen or immediately sonicated in 300 μl 1.0 N HCl. Chicks in the nonischemic control group were decapitated directly into liquid nitrogen for rapid inactivation of retinal metabolism. In some chicks from the nonischemic group, the external eyelids were surgically removed under CO_2 anesthesia prior to light or dark adaptation. This was done to hasten retinal freezing since skin has been reported to be the primary insulating factor which slows freezing rates in the brain (Swaab, 1971). Cyclic nucleotide levels in the retinal complexes of these chicks, however, were comparable to those of chicks sacrificed with intact eyelids. Subsequent experiments were therefore performed on chicks with intact eyelids.

Frozen nonischemic heads were transferred to a -30° C cryostat and, using prechilled instruments, pieces of retina-pigment epithelium-choroid (R-PE-C) as a complex were dissected from the eye. The PE was included with the retina to insure the acquisition of photoreceptor outer segments, which are deeply embedded in the overlying PE. The vascular choroid layer remained tightly attached to the PE in both fresh and frozen preparations. Frozen complexes were stored in liquid

nitrogen and then permanently inactivated by sonication in 300 μl ice cold 1.0 N HCl.

All homogenized samples (ischemic and nonischemic) were heated for 3 min to insure protein precipitation, centrifuged and the supernatant removed. Pellets were solubilized in 1.0 N NaOH and protein assayed according to the method of (Lowry et al., 1951) using BSA standard. Supernatants were neutralized, diluted and assayed for cAMP and cGMP content using an acetylated radioimmunoassay kit (Biochemical Technologies, Inc.). Cyclic nucleotide content is expressed as pmole cyclic nucleotide per mg R-PE-C protein.

Assay validity was established by reacting some samples and standard with commercial phosphodiesterase (PDE) 50 μg/ml) for various periods prior to assay. Sample levels of cAMP and cGMP were below detection following 1 h of PDE hydrolysis. The potential presence of interfering substances was tested by purifying some retinal extracts prior to assay on BioRad AG1-X8 (200-400 mesh) formate columns, using 1.0 N formic acid to elute cAMP and 4N formic acid to elute cGMP (Farber and Lolley, 1982). Both cAMP and cGMP levels were similar in purified and nonpurified retinal extracts, ruling out sample interference.

Effect of freezing on cAMP and cGMP in chick retina

Normally sighted White Leghorn chicks, 1-2 days posthatch, were light or dark-adapted for 2-3 hours and sacrificed by decapitation. Animals were enucleated and the R-PE-C complexes isolated from each eye. The R-PE-C complex from one eye was frozen in liquid nitrogen while, at the same time, the complex from the other eye was sonicated in ice cold 1.0 N HCl. Both the frozen and nonfrozen complexes were therefore exposed to identical ischemic durations, which averaged 150 sec in the light and 220 sec in the dark. Frozen complexes were subsequently sonicated in cold acid and samples assayed for cAMP and cGMP as described above.

cAMP PDE and cGMP PDE histochemistry

In order to estimate the retinal locations of cAMP and cGMP, the activity of the hydrolytic enzyme for each nucleotide was localized histochemically. Light-adapted carrier and rd chicks, 1-2 days posthatch, were sacrificed by decapitation. Eyecups were isolated and fixed in 0.2% glutaraldehyde, 2% paraformaldehyde in 0.1M Na cacodylate, 0.25M sucrose, pH 7.3 for 30-60 minutes at 4° C. Fixed eyes were frozen in liquid Freon and 10-20 μm frozen sections cut in a -20°C cryostat. Sections were preincubated 15 minutes, 24°C, pH 7.5 in 80mM tris maleate, 0.25M sucrose, 2mM $MgCl_2$ and approximately 115 units/ml 5'-nucleotidase from Crotalus adamanteus venom (Sigma). Sections were then incubated for 30 min at 37°C, pH 7.5, in TMS buffer containing 2mM $MgCl_2$, 3mM cAMP or 3mM cGMP, 60 units/ml nucleotidase and 3mM $CeCl_3$ as a capture reagent. After washing briefly, sections were placed in a 5mM Pb citrate secondary capture reagent solution for 5 min and washed. Reaction product was made visible at the light microscope level by staining the sections with 2% ammonium sulphide for 1 minute and coverslipped with glycerin.

cAMP and cGMP in microdissected layers of nonischemic chick R-PE-C

Sighted carrier chicks, 1 day posthatch, were light or dark-adapted for 2 to 3 h and sacrificed by decapitation into liquid nitrogen. Eyes were isolated from frozen heads in a -20°C cryostat and mounted on a cryostat chuck. 5-10 μm frozen sections of central R-PE-C were then cut tangentially through the posterior eye pole in order to maximize the width of the retinal layers. Frozen sections were freeze dried in a special drying assembly overnight at -35°C under a vacuum less than 0.1mm Hg. Freeze-dried sections were transferred a few at a time to a dissecting microscope contained within a humidity and draft controlled glove box. The choroid, pigment epithelium and individual retinal layers were identifiable in the freeze dried sections.

For these initial studies, sections were dissected into 3 layers; choroid, pigment epithelium with photoreceptor outer segments and all other retinal layers (retina minus outer segments). Layers were dissected freehand under 2X-4X magnification using knives made from 2mm razor blade shards and hair loops. Individual layers dissected from 15-20 sections were extracted with 100 μl ice cold 1.0 N HCl, homogenized on ice and centrifuged. Protein was measured in the pellet fraction using the (Lowry et al., 1951) method for microgram amounts of protein and cAMP and cGMP measured in the supernatant using a reduced volume radioimmunoassay.

Results

cGMP in sighted and blind retinal complexes--effects of light and ischemia

In baseline nonischemic RPEC's of sighted carrier chicks, light-adapted levels of cGMP averaged 8.57 ± 1.27 pmole/mg protein, (n=8). Levels of cGMP from dark-adapted chicks (10.53 ± 0.55, n=8) were significantly higher (p=.001) than light-adapted levels. Therefore, under nonischemic conditions, the cone-dominant retinal complexes of sighted chicks demonstrate light-induced decreases in cGMP levels.

In nonischemic rd RPEC's, levels of cGMP were significantly depressed compared to those in the sighted chick RPEC. Both light-adapted levels (1.23 ± 0.10, n=7) and dark-adapted levels (1.35 ± 0.35, n=11) were depressed by approximately 85%. Unlike sighted chicks, the nontransducing RPEC's of rd chicks showed no light-dark difference in cGMP levels.

Under ischemic conditions, cGMP levels in sighted chick RPEC's were significantly higher than nonischemic levels, in both the light (12.98 ± 3.95, n=6) and the dark (13.14 ± 1.86, n=5). Ischemic levels of cGMP tended to be more variable than nonischemic levels and, unlike nonischemic retinas, ischemic RPEC's demonstrated no significant light-dark difference in cGMP levels. The ischemic

durations tested for light-adapted RPEC's ranged from 90 to 270 seconds and from 90 to 300 seconds for dark-adapted complexes. There was, however, no apparent correlation between levels of cGMP and the duration of ischemia in these whole RPEC complexes.

Levels of cGMP in rd RPEC's were also significantly elevated under ischemic conditions. Ischemic light-adapted levels of cGMP averaged 5.60 ± 1.30 (n=6) and ischemic dark-adapted levels of cGMP averaged 6.28 ± 1.15 (n=5), both 3.5 times greater than nonischemic levels. As in the nonischemic state, no light-dark difference in cGMP levels occurred in the blind retinal complexes. Ischemic durations for rd RPEC's ranged from 80 to 185 seconds in the light and from 140 to 280 seconds in the dark. Again, there was no apparent correlation between levels of cGMP and the ischemic duration. The rise in ischemic levels of cGMP is thus not dependent on function.

cAMP in sighted and blind retinal complexes - effects of light and ischemia

In nonischemic sighted RPEC's, light-adapted levels of cAMP were similar to those of cGMP, 8.24 ± 1.59 pmole/mg protein, (n=8). Unlike cGMP, however, no light-dark difference in cAMP levels was apparent in nonischemic complexes (dark cAMP = 3.00 ± 2.08, n=10). Under nonischemic conditions, rd RPEC's also failed to demonstrate a light-dark difference in cAMP levels. However, both light-adapted levels (12.35 ± 1.58, n=7) and dark-adapted levels (11.38 ± 1.83, n=10) of cAMP were significantly higher in rd RPEC's than in sighted complexes.

cAMP levels in ischemic sighted RPEC's were approximately double those of nonischemic complexes, in both the light (17.96 + 1.46, n=5) and the dark (19.38 ± 3.17, n=5). rd levels of cAMP were elevated under ischemic conditions in the dark (18.42 ± 1.44, n=5) but not in the light (13.06 ± 1.55, n=5). In both carrier and rd complexes, there was no apparent correlation between cAMP levels and the duration of ischemia.

Comparison of frozen and nonfrozen samples having identical ischemic durations showed that in both the light and dark, cAMP as well as cGMP levels were comparable in frozen and nonfrozen RPEC's. Thus, the elevated levels of cAMP and cGMP we observed to be associated with ischemia are not an artifactual consequence of freeze-induced cyclic nucleotide depression occurring within the nonischemic samples.

Histochemical localization of cAMP and cGMP phosphodiesterase

For both cGMP PDE and cAMP PDE, the localization and intensity of reaction product were comparable in sighted and rd retinas. cGMP PDE activity was largely restricted to the photoreceptor layers of the retina, being highest in the outer segments. cAMP PDE activity was also present in the photoreceptors but was additionally concentrated in the inner plexiform layer of the retina. Both PDE's were more effectively inhibited by 2mM IBMX than by 4mM theophylline. Control sections, incubated without 5' nucleotidase or without substrate were devoid of reaction product. These results indicate that cGMP is concentrated within the photoreceptor outer segments of the cone-dominant chick retina, while cAMP is more evenly distributed between the inner and outer retina. Additionally, the results show that both cAMP PDE and cGMP PDE are present and capable of function in the blind rd retina.

cAMP and cGMP in microdissected layers of sighted chick RPEC

Preliminary results of 2 eyes from light-adapted sighted chicks indicate that a large proportion of cAMP in the RPEC complex is concentrated within the choroid layer (23.61 ± 8.27 pmole/mg protein). This cAMP is most likely contributed by the blood within this vascular layer. Levels of cAMP in the pigment epithelium plus outer segment (PE+OS) region averaged 10.50 ± 2.30, comparable to the levels in all other retinal layers combined (9.40 ± 2.61).

cAMP, therefore, appears not to be disproportionately concentrated within the cone outer segments of the chick retina.

cGMP, on the other hand, is greatly enriched within the PE+OS layer of the chick retina. PE+OS levels of cGMP (25.39 ± 8.81) were 5 times higher than choroidal levels (4.94 ± 3.25) and 3 times higher than levels in all other retinal layers combined (7.28 ± 2.58). This indicates that cGMP is concentrated within the cone and rod outer segments of the chick retina and that cone outer segment levels of cGMP are approximately double those of cAMP.

Discussion

To date, little has been learned concerning about cyclic nucleotides in the cone-dominant retina and the few published reports (DeVries et al., 1979, Farber et al., 1981, Farber et al., 1983) disagree as to the relative importance of cGMP and cAMP in cone transduction. Our results indicate that cGMP is concentrated within the photoreceptor outer segments of the cone-dominant chick retina and that this nucleotide plays an important role in the function of these cells. As in rod-dominant retinas (Goridis et al., 1974), light reduces cGMP levels in sighted chick retinas, a finding not previously reported in a cone-dominant species. This finding provides biochemical evidence in support of cGMP's role in regulating the open state of photosensitive channels in the outer segments of cone photoreceptors (Haynes and Yau, 1985, Cobbs et al., 1985). The severely depressed levels of cGMP and their lack of light modulation in the nontransducing retina of the rd chick further implicates cGMP in cone transduction.

cAMP, on the other hand, is not unduly concentrated within the outer segments of the chick retina and its RPEC levels are not tightly coupled to retinal excitation.

Previous experiments in our laboratory (Ruth et al, 1984) showed that glucose utilization in the chicken retina is coupled to photoreceptor function.

In the dark, when high levels of cGMP maintain the depolarized state of the photoreceptors, retinal glucose utilization is high. This large glucose demand supports the Na^+K^+ATPase pump in the photoreceptors which maintains its depolarizing dark current. Light stimulation, which reduces outer segment levels of cGMP and thereby decreases current flow, also reduces glucose utilization in the chick retina. The nontransducing rd chick retina, however, shows no dark-induced increase in glucose utilization, behaving as if it were always in the hyperpolarized or off state.

Since dark-adapted chick retinas have higher energy requirements, it was expected that ischemia would have a greater effect on cyclic nucleotide levels in dark-adapted retinas. Ischemia-induced increases in cGMP were, in fact, less in dark-adapted chick RPEC's than in the light. Additionally, rd chick RPEC's, which are metabolically depressed, showed a proportionately much larger ischemia-induced increase in cGMP than sighted chicks.

In the brain (Steiner et al., 1972, Lust and Passoneau, 1979), and in rod-dominant retinas (Orr et al., 1976, Mitzel et al., 1978) ischemia decreases levels of cGMP. The ischemia-induced increases in cGMP we observed in the chick retina may be a cone-specific phenomenon. Cellular analysis of ischemic changes in cGMP and energy metabolite levels will help clarify this finding.

Ischemia also increases cAMP in the chick retina, consistent with observations in brain (Steiner et al., 1972) and rod-dominant retinas (Orr et al., 1976, Mitzel et al., 1978). While whole RPEC levels of cAMP are doubled during 2-3 min exposure to ischemia, we do not yet know if these increases are occurring within the retina proper or in the cAMP-rich choroid. Microdissection experiments are currently being performed to examine ischemic changes in cyclic nucleotide levels within the various cell layers of the chick retina, as well as the energy state of the various layers before and after ischemic insult.

In summary, ischemia increases levels of both cGMP and cAMP in the cone-dominant chick retinal complex. The increases in cGMP are most likely associated with the photoreceptors, while the elevation in cAMP may involve both retina and choroid. These ischemia-induced changes in whole RPEC levels of cyclic nucleotide, however, do not appear to be coupled to the light-adapted or functional state of the cone-dominant chick retina.

Anticipated Studies for Second Year. We have prepared a sufficient number of dissected samples from light and dark-adapted sighted and blind nonischemic chicks for measurement of cAMP and cGMP, and are measuring the energy metabolite levels ATP and creatine phosphate in nonischemic RPEC complexes from central retina. Early this Fall we will complete our measurements of cyclic nucleotide and energy metabolite levels in nonischemic retinal layers of light and dark-adapted sighted and blind chicks. The objective of year 2 is to examine the effects of ischemia on cyclic nucleotide and energy metabolite levels in the cone-dominant chicken retina. Measurements will be performed on light and dark-adapted retinas from sighted and blind chicks exposed to various ischemic intervals. We anticipate few problems following the research plan for year 2, having overcome many of the technical difficulties associated with the microdissection technique during year 1 of the grant.

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